Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins

Yong Zhang[1,3,4], Xinsheng Gao[1,4], Leslie J. Saucedo[2], Binggen Ru[3], Bruce A. Edgar[2] and Duojia Pan[1]

Mutations in the *TSC1* or *TSC2* genes cause tuberous sclerosis, a benign tumour syndrome in humans[1,2]. Tsc2 possesses a domain that shares homology with the GTPase-activating protein (GAP) domain of Rap1-GAP[2], suggesting that a GTPase might be the physiological target of Tsc2. Here we show that the small GTPase Rheb (Ras homologue enriched in brain) is a direct target of Tsc2 GAP activity both *in vivo* and *in vitro*. Point mutations in the GAP domain of Tsc2 disrupted its ability to regulate Rheb without affecting the ability of Tsc2 to form a complex with Tsc1. Our studies identify Rheb as a molecular target of the TSC tumour suppressors.

TSC1 and *TSC2* were initially discovered as tumour suppressor genes mutated in tuberous sclerosis, a human syndrome characterized by the widespread development of benign tumours termed harmatomas[1,2]. *TSC2* encodes a putative GAP protein, whereas *TSC1* encodes a novel protein containing two coiled-coil domains[1,2]. Studies of *Drosophila melanogaster TSC1* and *TSC2* homologues identified a specific function for *TSC1–TSC2* in the control of cell growth, with loss of *TSC1–TSC2* resulting in increases in cell size[3–5]. Recent studies further suggested that Tsc1–Tsc2 antagonizes the amino-acid–TOR signalling pathway, which normally couples amino-acid availability to S6 Kinase (S6K) activation, translation initiation and cell growth[6–9]. Strikingly, loss of *Drosophila TSC1–TSC2* results in a TOR-dependent increase of S6K activity that is resistant to amino-acid starvation[6].

Despite these new advances, the biochemical activity of the Tsc1–Tsc2 complex remains unknown. Tsc2 possesses a domain homologous to Rap1-GAP[2]. The GAP homology domain of Tsc2 is important for its function, and mis-sense mutations of this domain were identified in a high proportion of TSC patients[10]. These observations suggest that an unknown small GTPase might be the direct target of Tsc2. We set out to identify the target GTPase of Tsc2-GAP using an RNAi-based screen in *Drosophila* S2 cells (Supplemental Information). We reasoned that this putative GTPase should be expressed in S2 cells and that RNAi of this GTPase should result in downregulation of S6K-Thr 398 phosphorylation, a phenotype opposite to that caused by *Tsc2* RNAi[6]. During the course of the RNAi screen, genetic studies implicated the small GTPase Rheb as a potential target of Tsc2 (refs 11, 12). In S2 cells, RNAi inhibition of *Rheb*[11], but not any of the other 17 GTPases tested so far (see Supplementary Information, Table 1), abolished S6K-Thr 398 phosphorylation, as predicted for a Tsc2 GAP substrate. Among the 17 GTPases screened were Rab5 and Rap1, two proteins previously implicated as TSC2 GAP substrates from *in vitro* studies[13,14], suggesting that Rab5 and Rap1 are improbable physiological substrates of Tsc2. The highly specific effect of *Rheb* RNAi on S6K phosphorylation suggests that Rheb might be the physiological substrate of TSC2 GAP activity.

Rheb is an evolutionarily conserved small GTPase that is found from yeast to mammals[15]. Unlike Ras and most other Ras superfamily GTPases, Rheb has an arginine at the third residue of the G1 box (residue 15 of mammalian Rheb) instead of glycine[15]. Rheb is unique, compared with many small GTPases, in that it exists in a highly activated state in mammalian cells[16]. Studies of mammalian Rheb further implicated the existence of a Rheb-GAP that is normally present at relatively limiting concentrations, as overexpression of Rheb results in a progressive increase in the proportion of Rheb in the active GTP-bound state[16]. Genetic analyses in *Drosophila* support a model in which Tsc2 functions as a Rheb-GAP[3–5,11,12]. These studies also suggest that similarly to mammalian cells, Tsc2, the putative Rheb-GAP, is normally present in limiting concentrations in *Drosophila*, as overexpression of wild-type Rheb results in an activated phenotype[11,12] and overexpression of Tsc2 (together with Tsc1) results in the opposite phenotype[3–5].

To test directly whether Rheb is a physiological substrate of Tsc2 GAP activity, we asked if Tsc2 could regulate Rheb *in vivo*. Rheb, similarly to other small GTPases, cycles between an active GTP-bound form and an inactive GDP-bound form. Thus, the steady state GTP/GDP-loading status of Rheb can be used as a measurement of its *in vivo* activity. We adapted an *in vivo* labelling procedure[17] to analyse the steady-state GTP/GDP-binding status of Rheb. *Drosophila* S2 cells expressing Myc-tagged Rheb were labelled with ^{32}P-orthophosphate. Rheb protein was then purified by immunoprecipitation and Rheb-associated GTP/GDP was analysed by thin-layer chromatography (TLC) on polyethyleneimine (PEI) cellulose plates. In wild-type S2 cells, Rheb bound preferentially to GTP (Fig. 1a), in agreement with previous studies of mammalian Rheb[16]. In addition, co-overexpression of Tsc1 and Tsc2 resulted in a marked decrease (approximately eightfold) in the ratio of GTP to GDP bound on Rheb. Interestingly, overexpression of Tsc2 alone had much weaker effect on GTP:GDP ratio. This observation is consistent with previous studies in *Drosophila*, which showed that co-overexpression of Tsc1 and Tsc2, but not either gene alone, resulted in growth inhibition[3–5]. The weaker effect of Tsc2 alone on Rheb GTP loading is caused, at least in part, by the lower level of Tsc2 when expressed alone, as compared with Tsc1 co-expression (Fig. 1a). Mutual stabilization between *Drosophila* Tsc1 and Tsc2 has been documented previously[6].

To demonstrate that the effect of Tsc1–Tsc2 overexpression on Rheb GTP loading was caused by the GAP activity of Tsc2, we performed

[1]Department of Physiology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390-9040, USA. [2]Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, WA 98109, USA. [3]Department of Biochemistry, Peking University, Beijing 100871, China. [4]These authors contributed equally to this work. Correspondence should be addressed to D.P. (dpan@mednet.swmed.edu).

Exhibit A (5 pages total)

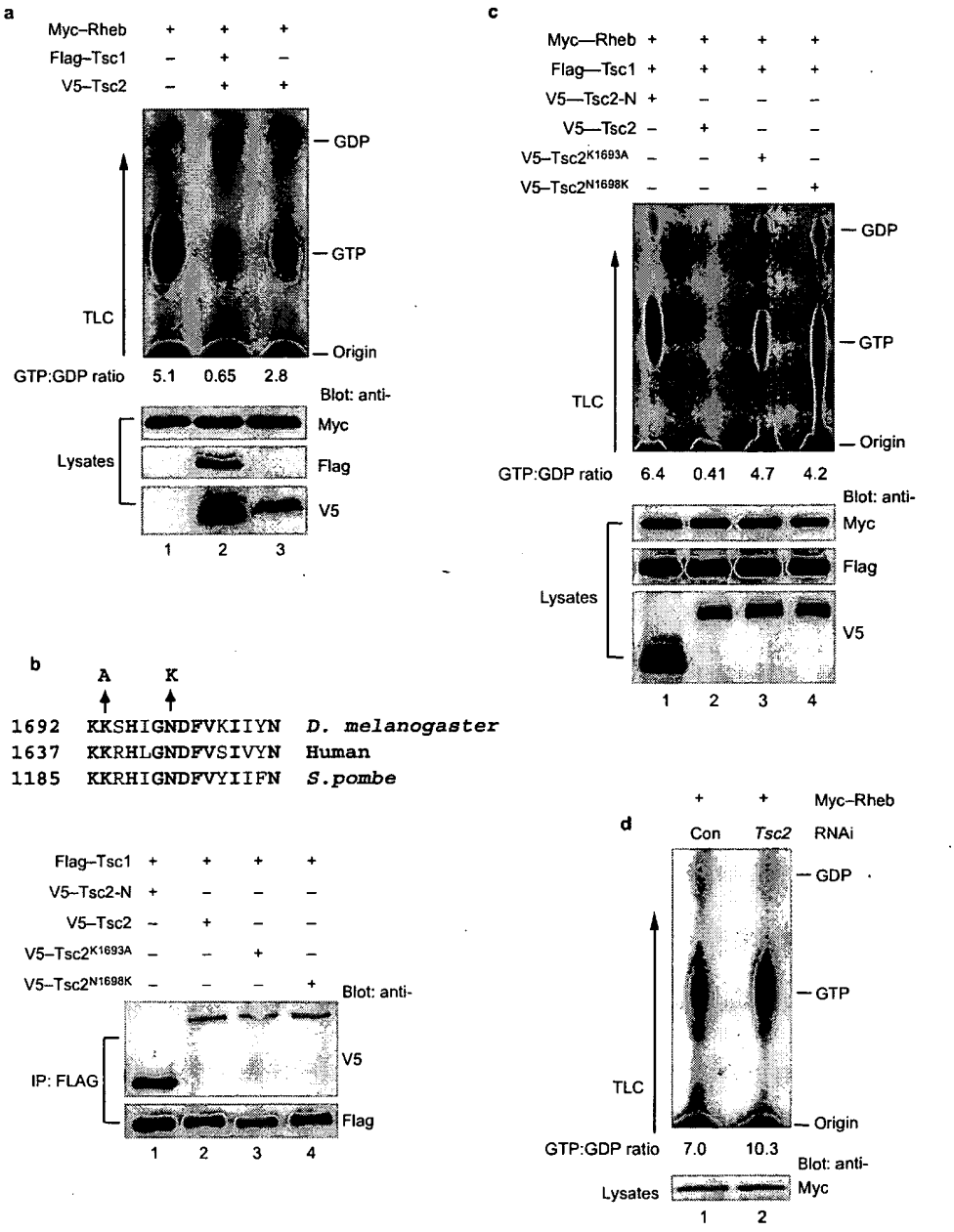

Figure 1 Tsc2 regulates Rheb activity *in vivo*. **(a)** Co-expression of Tsc1 and Tsc2 reduces GTP loading of Rheb *in vivo*. GTP loading of Rheb was analysed in S2 cells expressing the indicated plasmids. The origin of chromatography, the migration of GTP and GDP and their relative ratio are indicated. A portion of cell lysate was taken before immunoprecipitation to probe for expression of each construct (lysates). **(b)** A schematic representation of a conserved region within the Tsc2 GAP domain and point mutations used in this study (top). $Tsc2^{K1693A}$ or $Tsc2^{N1698K}$ do not affect the association between Tsc2 and Tsc1 (bottom). Anti-Flag immunoprecipitates from S2 cells expressing Flag–Tsc1 and various V5-tagged Tsc2 constructs were analysed for the presence of Tsc2 (top gel; anti-V5) and Tsc1 (bottom gel; anti-Flag). **(c)** Mutations in the GAP domain of Tsc2 abolish its ability to regulate Rheb *in vivo*. GTP loading of Rheb was analysed in S2 cells expressing the indicated plasmids. The expression level of each construct was also analysed (lysates). **(d)** RNAi of *Tsc2* increases GTP loading of Rheb. GTP loading of Rheb was analysed in S2 cells treated with control or *Tsc2* double-stranded RNA (dsRNA).

similar *in vivo* labelling experiments with Tsc2 variants carrying point mutations in the GAP domain. The mutations $Tsc2^{K1693A}$ and $Tsc2^{N1698K}$ changed residues in the GAP domain that are conserved in *Drosophila*, human and a probable *Schizosaccharomyces pombe* Tsc2 homologue[18] (Fig. 1b). In addition, a mutation analogous to $Tsc2^{K1693A}$ has been shown to abolish Rap1-GAP activity[19], whereas $Tsc2^{N1698K}$ mimics a disease-causing mutation in human TSC patients[10]. We also examined the activity of Tsc2-N, a construct that

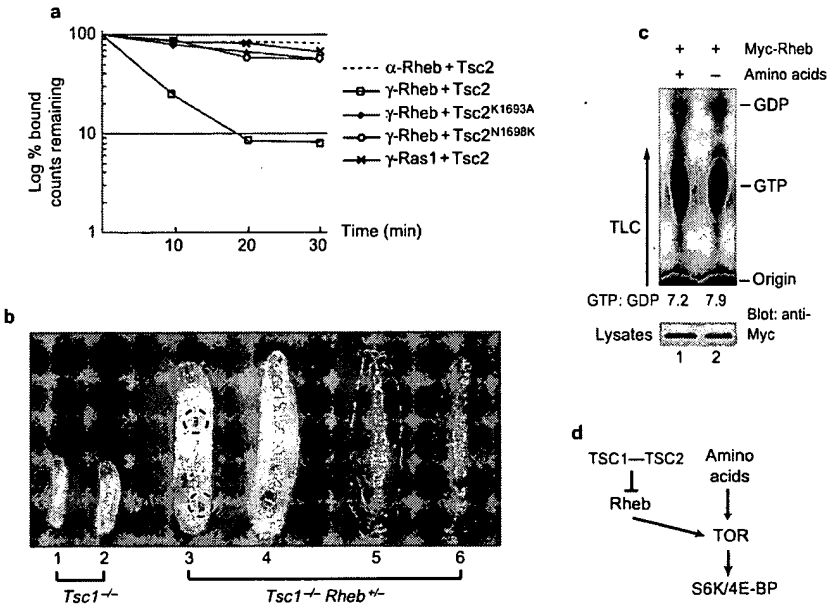

Figure 2 *In vitro* Rheb GAP activity of Tsc2 and genetic interaction between *Tsc1* and *Rheb*. (**a**) GST fusion proteins containing Tsc2 GAP domain, or the corresponding K1693A or N1698K mutations were tested for *in vitro* GAP activity using γ-^{32}P-GTP-loaded Rheb, γ-^{32}P-GTP-loaded Ras1 or α-^{32}P-GTP-loaded Rheb, as indicated. The fraction of radioactive counts that remained bound to Rheb at different times was determined by nitrocellulose filter assay[20] and plotted on a $\log_{10}$/time chart. (**b**) Heterozygosity of *Rheb* partially rescues the lethality of *Tsc1* mutants. Comparison of animals of different genotypes: $Tsc1^{29}/Tsc1^{29}$ (1 and 2, arresting as second-instar larvae), $Tsc1^{29}$ $Rheb^{P\Delta1}/Tsc1^{29}$ (3 and 4, late third-instar larvae; 5 and 6, pupae). Often the *Tsc1* mutants rescued by heterozygosity of *Rheb* contained melanotic tumours (two such examples are marked by dotted circles in animal 3). (**c**) Rheb activity is not regulated by amino-acid sufficiency. S2 cells expressing Myc–Rheb were incubated in complete medium (lane 1) or amino-acid-free medium (lane 2) for 5 h. The ratio of GTP:GDP bound on Rheb was not significantly changed. (**d**) Proposed model of Tsc–Rheb function in TOR signalling.

contains just the amino-terminal half of Tsc2 and thus lacks the carboxy-terminal GAP domain. Tsc2-N could associate with Tsc1 normally[3], but did not interact with Rheb in co-immunoprecipitation assays (data not shown). Similarly to Tsc2-N, neither $Tsc2^{K1693A}$ nor $Tsc2^{N1698K}$ affected the ability of Tsc2 to associate with Tsc1 (Fig. 1b). Despite their ability to associate with Tsc1, these mutants all abolished the effect of Tsc1–Tsc2 overexpression on Rheb GTP loading (Fig. 1c). Complementary to the results from Tsc1–Tsc2 overexpression, RNAi of *Tsc2* increased the ratio of GTP:GDP bound to Rheb (Fig. 1d). The smaller change in GTP:GDP ratio after *Tsc2* RNAi, compared with Tsc1–Tsc2 overexpression, is not surprising given that Rheb is already at a relatively active state in wild-type cells. Taken together, these results provide strong evidence that Rheb is a physiological target of Tsc2 GAP activity.

To test whether Rheb is a direct substrate of Tsc2 GAP *in vitro*, we tested a fusion protein of glutathione *S*-transferase (GST) and the Tsc2 GAP domain against GTP-loaded Rheb protein using a nitrocellulose filter assay[20]. α-^{32}P-GTP- or γ-^{32}P-GTP-loaded GST–Rheb was incubated with GST–Tsc2 and the remaining radioactive GTP bound on Rheb was measured at different time intervals. GST–Tsc2 resulted in a dramatic decrease of Rheb-associated radioactive counts when γ-^{32}P-GTP, but not α-^{32}P-GTP, was used in the assay (Fig. 2a). Thus, Tsc2 functions as a Rheb GAP *in vitro*. This GAP activity is highly specific, and no activity was detected using *Drosophila* Ras1, the closest relative of Rheb among all GTPases, as a substrate (Fig. 2a). In addition, the K1693A or the N1698K point mutation abrogated the *in vitro* GAP activity of Tsc2 towards Rheb (Fig. 2a). These results provide further evidence that Tsc2 functions as a Rheb GAP.

The data presented so far suggest a model in which the tuberous sclerosis tumour suppressor proteins negatively regulate Rheb through the Rheb GAP activity of Tsc2. To further substantiate this model, we tested whether there are any genetic interactions between *TSC1–TSC2* and *Rheb*. Flies homozygous for a null allele of *TSC1*, $TSC1^{29}$, do not survive beyond the second-instar larval stage[3]. Strikingly, the lethality of *TSC1* null animals was partially rescued by removing one of the two copies of *Rheb* gene from the diploid genome: 61% ($n = 160$) of $TSC1^{29}$ homozygotes that were also heterozygous for a null allele of *Rheb*, $Rheb^{P\Delta1}$ (ref. 11), survived to third-instar larval stage, and 21% of the third-instar survivors continued development and arrested at the pupal stage (Fig. 2b). Such dose-sensitive interactions are reminiscent of those observed between *TSC1–TSC2* and *TOR*[6], further supporting the model that Tsc1–Tsc2 negatively regulates Rheb during cell growth.

Finally, we investigated how the Tsc–Rheb pathway interacts with the amino acid–TOR–S6K signalling network. Tsc and Rheb could either function as obligatory components between amino acids and TOR in a linear amino-acid sensing pathway, or in a parallel pathway that converges on TOR[6]. The former, but not the latter, model predicts that the activity of Rheb is dependent on the presence of amino acids. As shown in Fig. 2c, the ratio of GTP:GDP bound to Rheb was not reduced after 5 h of amino-acid starvation. Thus, we favour a model in which TSC and Rheb function in a parallel pathway that converges on TOR (Fig. 2d). According to this model, loss of Tsc1–Tsc2 or ectopic activation of Rheb results in constitutive activation of TOR, which bypasses the requirement for amino acids and renders S6K activity resistant to amino-acid starvation. How Rheb signals to TOR will be an important question for future investigation.

In summary, we have identified the small GTPase Rheb as a direct target of the tuberous sclerosis tumour suppressor proteins. We demonstrate that wild-type Tsc2, but not mutant Tsc2 carrying point mutations in the GAP domain, shows GAP activity towards Rheb both *in vitro* and *in vivo*. The importance of Tsc2's GAP activity is further supported by the high proportion of mis-sense mutations localized to the Tsc2 GAP domain among TSC patients[10]. Thus, the Tsc2 tumour suppressor functions as a Rheb-GAP in an analogous way to the neurofibromin (NF1) tumour suppressor as a Ras-GAP. Our studies suggest that Rheb represents a novel target for therapeutic intervention in the TSC disease. The identification of a small GTPase as the direct target of the TSC tumour suppressors further implicates the existence of activators of GTPases, such as guanine nucleotide-exchange factors (GEFs), as potential regulators of this disease pathway. Identification of the putative Rheb-GEF represents an important goal for the next phase of TSC research. □

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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S2 cell culture, expression constructs and immunoprecipitation. S2 cells were propagated in *Drosophila* serum-free medium (Invitrogen, Carlsbad, CA), as described previously[6]. Myc–Rheb was made in pAc5.1/V5-HisB vector (Invitrogen) by fusing the Myc epitope (MEQKLISEEDLNE) to the amino terminus of full-length Rheb. Point mutations in Tsc2 were introduced using QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). Constructs for epitope-tagged Tsc1 and Tsc2, and immunoprecipitation procedures have been described previously[3]. Antibodies against Myc, V5 and Flag epitopes were from Santa Cruz Biotechnology (Santa Cruz, CA), Invitrogen and Sigma (St Louis, MO), respectively.

***In vivo* labelling of Rheb.** *In vivo* labelling of Rheb was adapted from ref. 17. Briefly, 3×10^6 S2 cells per 60-mm plate were transfected with Myc–Rheb together with indicated combination of Tsc1 and Tsc2 plasmids using the Effectene reagent (Qiagen, Valencia, CA). After 48 h, cells were rinsed once and incubated overnight in phosphate-depleted Schneider's *Drosophila* Medium containing 250 μCi ml^{-1} ^{32}P-orthophosphate (HCl-free; Amersham Pharmacia Biotech, Piscataway, NJ). Labelled cells were washed once with PBS and lysed for 30 min in 0.5 ml ice-cold lysis buffer (10 mM Tris-HCl at pH 7.4, 150 mM sodium chloride, 1% Triton X-100, 0.2 mM EDTA, 0.2 mM EGTA, 10 mM magnesium chloride, 25 mM sodium fluoride, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM GDP, 1 mM GTP and 1 mM phenyl methylsulphonyl fluoride (PMSF)). Cell lysates were centrifuged and one-tenth of each supernatant was taken out for western blotting. The remaining lysates were incubated with 2 μg of anti-Myc monoclonal antibody (Santa Cruz) and 20 μl of Protein G–agarose beads (Amersham Pharmacia Biotech) at 4 °C for 4 h. Immunoprecipitates were washed three times with lysis buffer and eluted with 20 μl of elution buffer (75 mM KH_2PO_4 at pH 3.4, 5 mM EDTA, 0.5 mM GTP and 0.5 mM GDP) at 85 °C for 3 min. Elutions were spotted on a PEI cellulose TLC plate (Selecto Scientific, Suwanee, GA), resolved in 1 M KH_2PO_4 at pH 3.4 for 90 min and detected by autoradiography.

Experiments involving amino-acid starvation were performed as above, except that ^{32}P-orthophosphate-labelled cells were transferred to complete Schneider's *Drosophila* Medium or amino-acid-free medium[6] for an additional 5 h before cell lysis. Experiments involving RNAi were also performed as above, except that S2 cells were cotransfected with Myc–Rheb and *Tsc2* dsRNA. dsRNA of the mammalian *CYP7A1* gene was used as controls in RNAi experiments. It should be noted that the GTP:GDP ratio of Rheb under normal conditions (that is, without Tsc1/Tsc2 overexpression or RNAi) varies between 5.1 and 7.2 in different experiments (Figs. 1a, c, d and 2c). This variability might be caused by the varying amounts of Rheb expressed in different experiments. It is known that Rheb expression levels affect its activation state[16].

***In vitro* GAP assay.** pGEX-4T-1 vector was used to express GST fusion proteins containing Rheb, Tsc2-GAP and Tsc2-GAP point mutants. The GST fusion of *Drosophila* Ras1 was a gift of M. White. GST–Tsc2-GAP and point mutants included amino acid 1384–1847 of Tsc2. GST fusion proteins were expressed in *Escherichia coli* BL21 cells and purified using standard procedures (Amersham Pharmacia Biotech). A nitrocellulose filter assay[20] was used to measure *in vitro* GAP activity. Briefly, 5 μg of GST–Rheb or GST–Ras1 was incubated with 10 μCi of γ-^{32}P-GTP (or α-^{32}P-GTP if indicated) in 40 μl of loading buffer (20 mM Tris-HCl at pH 7.5, 25 mM sodium chloride, 0.1 M dithiothreitol and 5 mM EDTA) for 30 min at 30 °C. The reaction was stopped by adding ice-cold magnesium chloride to a final concentration of 5 mM and incubating on ice for 3 min. GTP-loaded protein (10 μl) was diluted in 50 μl GAP assay buffer (loading buffer containing 1 mM GTP and 5 mM magnesium chloride) containing 2 μg GST–Tsc2-GAP protein. At 0, 10, 20 and 30 min, 10-μl aliquots were diluted in 1 ml of ice-cold washing buffer (50 mM Tris-HCl at pH 7.5, 50 mM sodium chloride and 5 mM magnesium chloride), passed through nitrocellulose filters and washed with 12 ml washing buffer. The filters were dried and quantified by scintillation counting.